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# a1/EBP: a Leucine Zipper Protein That Binds CCAAT/Enhancer Elements in the Avian Leukosis Virus Long Terminal Repeat Enhancer

W. J. BOWERS AND A. RUDDELL*

*Department of Microbiology and Immunology and Cancer Center, University of Rochester Medical Center, Rochester, New York 14642*

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**Avian leukosis virus (ALV) induces bursal lymphoma in chickens after integration of proviral long terminal repeat (LTR) enhancer sequences next to the c-*myc* proto-oncogene. Labile LTR-binding proteins appear to be essential for c-*myc* hyperexpression, since both LTR-enhanced transcription and the activities of LTR-binding proteins are specifically decreased after inhibition of protein synthesis (A. Ruddell, M. Linial, W. Schubach, and M. Groudine, J. Virol. 62:2728–2735, 1988). This lability is restricted to hematopoietic cells from ALV-susceptible chicken strains, suggesting that the labile proteins play an important role in lymphomagenesis. The major labile activity binding to the a1 LTR region (A. Ruddell, M. Linial, and M. Groudine, Mol. Cell. Biol. 12:5660–5668, 1989) was purified from bursal lymphoma cells by conventional and oligonucleotide affinity chromatography, yielding three proteins of 35, 40, and 42 kDa. More than one of these species binds the a1 LTR region, as judged by gel shift analysis. A gene encoding an a1-binding protein (designated a1/EBP) was cloned by screening a bursal lymphoma cDNA library for fusion proteins binding the a1 LTR site. DNase I footprinting and gel shift assays indicate that the a1/EBP fusion protein binds multiple LTR CCAAT/enhancer elements in a pattern similar to that of the purified B-cell protein. DNA sequence analysis shows that this 2.2-kb cDNA encodes a 209-amino-acid open reading frame containing carboxy-terminal basic and leucine zipper motifs, indicating that a1/EBP encodes a novel member of the leucine zipper family of transcription factors.**

The oncogenic potential of retroviruses causing nonacute diseases appears to be regulated by the U3 long terminal repeat (LTR) enhancer as well as by the viral *env* gene (6, 22, 57). Transcription factor binding to the LTR enhancer may regulate the frequency and tissue specificity of tumor induction. For example, the oncogenicity of murine leukemia virus strain SL3-3 is influenced by mutations in LTR enhancer protein-binding sites (19, 30). Thymic lymphoma or erythroleukemia induction by Moloney or Friend murine leukemia virus, respectively, is largely determined by differences in multiple protein-binding sites in the LTR enhancer (16, 56).

Avian leukosis virus (ALV) provides a well-characterized system for analysis of the role of transcription factors in tissue-specific transformation. ALV induces B-cell lymphoma in chickens after the rare integration of a proviral U3 LTR enhancer next to the c-*myc* proto-oncogene (39, 42). This LTR deregulation of c-*myc* expression, giving up to 100-fold increases in c-*myc* expression, is an important early determinant of lymphomagenesis (12, 40). The LTR enhancer is required for tumor induction, as endogenous ALV retroviruses lack complete enhancer elements and rarely induce lymphoma (6, 46, 61). Host cell factors also play an important role in lymphomagenesis, as some strains of chickens are resistant to ALV lymphoma (2, 14). Transplantation experiments have demonstrated that the target pre-B cells encode the resistance phenotype (45). The nature of the host factors mediating resistance is not known, as the patterns of ALV infection, integration, and expression are similar in susceptible and resistant strains (2, 14). Moreover, viral expression is similar in many cell types, including immature or mature bursal cells (50), even though tumor induction is specific for immature B cells. Thus, the factors regulating ALV pre-B-cell susceptibility do not act simply by restricting high-level viral protein or oncogene expression to target pre-B cells.

We have identified one characteristic of ALV LTR enhancement which does correlate with pre-B-cell susceptibility to tumor induction. LTR-enhanced c-*myc* and viral gene transcription is specifically decreased 10- to 15-fold after inhibition of protein synthesis in bursal lymphoma cells, while LTR-enhanced transcription is unaffected by protein synthesis inhibition in infected T cells or embryo fibroblasts (31). These findings suggest that labile or short-lived proteins regulate LTR enhancement in B cells. This lability is restricted to immature hematopoietic cells of ALV-susceptible chicken strains, while LTR-enhanced transcription is stable in all tissues of ALV-resistant chicken strains (50). The correlation of labile LTR enhancement with pre-B-cell susceptibility suggests that this lability is important in ALV lymphomagenesis. Labile LTR enhancement could influence c-*myc* hyperexpression in a manner essential for tumor induction. For example, c-*myc* mRNA and protein are very short-lived (9, 20); consequently, developmental down-regulation of labile LTR enhancement could transiently down-regulate c-*myc* expression, improving the survival of target pre-B cells (50). This labile down-regulation could reduce the cytotoxicity of hyperexpressed c-*myc* (11, 57, 59) or could allow bursal differentiation events required for lymphomagenesis (8, 45).

We previously analyzed nuclear proteins binding to the ALV LTR enhancer to determine whether labile binding proteins could regulate LTR-enhanced transcription in pre-B cells. Five LTR enhancer-binding proteins were identified by gel shift and footprinting analyses of nuclear proteins from

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* Corresponding author.

bursal lymphoma cells (49, 50). Three of the proteins (a1, a3, and b*) are specifically labile in pre-B cells, while they are stable or not expressed in other cell types. These findings suggest that binding of the labile proteins is essential for high rates of LTR-enhanced transcription in B cells. The major a1 protein-binding site contains multiple CCAAT/enhancer elements, which are protected in DNase I footprinting assays with B-cell nuclear extracts or with partially purified B-cell proteins. CCAAT/enhancer elements are found in many viral and cellular gene enhancers (7, 23), and proteins binding to these regions can activate transcription. Several proteins that bind to these elements have been identified. For example, the C/EBP protein binds many CCAAT/enhancer elements (23, 51) and can activate or repress transcription (13, 44). The Ig/EBP and NF/IL-6 proteins bind CCAAT/enhancer elements in the immunoglobulin (Ig) heavy-chain and interleukin-6 (IL-6) gene enhancers, respectively (1, 48). These proteins belong to a family of leucine zipper transcription factors which feature conserved DNA-binding regions enriched in basic amino acids and conserved leucine heptad repeats which allow formation of homodimers or heterodimers with other leucine zipper proteins (29, 48). The C/EBP and Ig/EBP proteins also bind CCAAT/enhancer elements in the Rous sarcoma virus (RSV) LTR (48, 51), which is very similar to the ALV LTR (3). Such proteins could be involved in labile LTR binding and enhancement if they are in fact expressed in avian pre-B cells.

We have purified the a1 LTR-binding activity from chicken bursal lymphoma cells. Three proteins of 35 to 42 kDa are enriched after purification, and more than one of these species appear to encode sequence-specific a1-binding activity. These could represent distinct proteins or one protein that is differentially modified. To further characterize these proteins, a λgt11 cDNA library from bursal lymphoma cells was screened for cDNAs encoding a1 LTR-binding activity. One cDNA clone that encodes an a1 LTR-binding protein closely related to the Ig/EBP leucine zipper factor (48) was obtained. Analysis of this cloned gene will determine whether it encodes a labile protein regulating LTR enhancement and susceptibility to ALV lymphomagenesis.

## MATERIALS AND METHODS

**B-cell protein purification.** S13 bursal lymphoma cells were grown in spinner flasks (Bellco) in RP-9 medium (Dulbecco modified Eagle medium supplemented with 5% calf serum, 1% heat-inactivated chicken serum, and tryptose phosphate broth [GIBCO Laboratories]). Cells were harvested by centrifugation at 2,000 × *g*, washed in phosphate-buffered saline (140 mM NaCl, 11 mM KCl, 1.5 mM $KH_2PO_4$, 6.5 mM $Na_2HPO_4$, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$), and centrifuged again. Cell pellets were used immediately or were frozen in liquid nitrogen and stored at −70°C. Nuclear extracts were prepared by 0.5 M NaCl treatment of purified nuclei as previously described (50). Extracts were separated by S-300 Sepharose chromatography (Pharmacia) in buffer A (10% glycerol, 50 mM KCl, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol [DTT], 1 mM sodium metabisulfite, 0.2 ng of leupeptin per ml, 0.2 ng of pepstatin per ml 1 U of aprotinin HCl per ml, 1 mM phenylmethylsulfonyl fluoride [PMSF] [all from Sigma]). Column fractions were analyzed for protein content by the Bradford assay (5) and for a1 LTR-binding activity by a gel shift assay (see below). Fractions containing a1-binding activity were pooled, heated at 85°C for 10 min, and then centrifuged for 10 min at 12,000 × *g* to remove insoluble protein. The heat-purified supernatant was applied to a concatenated a1 oligonucleotide-agarose column (prepared as described by Kadonaga et al. [24]) in 0.075 M KCl–buffer B [50 mM HEPES (pH 7.9), 20% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 1 mM sodium metabisulfite, 10 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia), 0.2 ng of leupeptin per ml, 0.2 ng of pepstatin per ml, 1 U of aprotinin HCl per ml, 1 mM PMSF], and bound protein was eluted with 0.6 M KCl–buffer B.

**Gel shift assay.** Protein was incubated with 5,000 cpm (approximately 0.1 ng) of the $^{32}P$-labeled a1 oligonucleotide probe in gel shift buffer (10 mM Tris HCl, [pH 8.0], 50 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT) and 0.1 mg of poly(dI-dC) · poly(dI-dC). The 15-μl reaction mixtures were incubated at room temperature for 20 min and then electrophoresed on 4% polyacrylamide gels in TAE buffer (6.7 mM Tris, 3.3 mM sodium acetate, 1 mM EDTA [pH 7.5]) at 30 mA for 1 h as previously described (50). In some assays, unlabeled a1 or b oligonucleotide was added as a competitor (49). The double-stranded a1 oligonucleotide probe sequence is 5′-GGGAAATGTAGTCTTATGCAATACTCTAA-3′/5′-TTCCCTTAGAGTATTGCATAAGACTACAT-3′); the b oligonucleotide probe sequence is 5′-AAGGAGAGAAAAA GTACCGTGCATG-3′/5′-CATGCACGGTACTTTTTCTCT CCTT-3′.

**SDS-PAGE.** Protein samples were diluted to 0.25 ml and were precipitated with 10 μg of bovine lactoglobulin carrier (Sigma) in 4 volumes of cold acetone by chilling on dry ice for 30 min. Precipitates were centrifuged at 12,000 × *g* for 20 min, rinsed with cold 80% acetone, recentrifuged, and resuspended in sodium dodecyl sulfate (SDS) sample buffer (27). Samples were subjected to electrophoresis on 10% polyacrylamide–SDS-gels (SDS-PAGE) alongside molecular weight markers (Amersham) and then subjected to fixation in 50% methanol–0.04% formaldehyde and silver staining (38).

**Protein renaturation from SDS-polyacrylamide gels.** Three hundred micrograms of bursal lymphoma protein (purified by S-300 chromatography and heat treatment) was precipitated with 80% acetone, resuspended in SDS sample buffer, and separated by SDS-PAGE as described above. Ten slices were excised from the gel lane and were eluted overnight at 22°C in 0.4 ml of buffer C (40 μg of bovine serum albumin [BSA; Miles Biochemical] per ml, 50 mM Tris HCl [pH 7.5], 0.1 mM EDTA, 0.1% SDS, 5 mM DTT, 0.1 M NaCl [21]). The eluate was precipitated with 80% acetone as described above and then renatured in 40 μl of buffer D (1.5 mg of BSA per ml, 20% glycerol, 20 mM HEPES, [pH 7.9], 0.05 M NaCl, 0.1% Nonidet P-40, 0.1 mM EDTA, 0.5 mM DTT, 1 mM sodium metabisulfite, 0.2 ng of leupeptin per ml, 0.2 ng of pepstatin per ml, 1 U of aprotinin HCl per ml, 0.5 mM PMSF) at 4°C overnight with gentle rocking. Aliquots were analyzed by gel shift assay with or without an unlabeled competitor as described above.

**DNase I footprinting assay.** The 245-bp *Mst*II-*Eco*RI fragment of an ALV LTR subcloned from BK25 bursal lymphoma cells was $^{32}P$ end labeled as described previously (49). Gel shift reactions with $^{32}P$-labeled ALV LTR and 0.01 to 2 μg of a1/EBP fusion protein were incubated with DNase I (bovine pancreatic; Sigma) at 0.01 to 0.05 μg/ml in 5 mM $CaCl_2$–5 mM $MgCl_2$ (15). Following a 1-min DNase I treatment, the reactions were stopped with TENS (10 mM Tris HCl [pH 8.0], 1 mM EDTA, 0.1 M NaCl, 0.1% SDS) solution, proteinase K treated, and precipitated with 70% ethanol–2 M ammonium acetate. Samples were resolved on

8 M urea–8% polyacrylamide gels in parallel with the corresponding A+G sequence reactions (35).

**Isolation of a recombinant a1/EBP clone.** The λgt11 screening technique of Singh et al. (54) was used to identify cDNAs encoding a1-binding proteins. Poly(A)$^+$ RNA was purified from S13 bursal lymphoma cells by guanidinium isothiocyanate-cesium trifluoroacetate ultracentrifugation (41) and then two rounds of oligo(dT)-cellulose chromatography (Bethesda Research Laboratories). First-strand cDNA was prepared from the RNA by using murine leukemia virus reverse transcriptase (Pharmacia), and second-strand cDNA was synthesized by using Klenow DNA polymerase (18); *Eco*RI-*Not*I linkers were then ligated and cloned into the *Eco*RI site of λgt11 (60). The primary phage library ($1.5 \times 10^6$ phage) was plated on *Escherichia coli* Y1090, and isopropylthiogalactopyranoside (IPTG)-induced fusion proteins were transferred to nitrocellulose. Duplicate filters were screened for binding to the $^{32}$P-labeled concatenated a1 oligonucleotide probe, which was prepared by ligation and nick translation. Positive phage were replated and successively screened twice for a1 oligonucleotide-binding activity.

**DNA sequence analysis.** The a1/EBP cDNA insert was isolated from purified λgt11 phage DNA by *Not*I digestion and was subcloned into the *Not*I site of the Bluescribe plasmid (Stratagene) for DNA sequence analysis. Plasmids were purified by cesium chloride-ethidium bromide ultracentrifugation, and the sequences of both denatured plasmid strands were determined by dideoxy sequencing, using vector or cDNA-specific primers. Deaza-GTP and dITP sequence reactions were compared to resolve compressed regions (37). DNA sequences were analyzed by using Genetics Computer Group computer programs (10, 43).

**Bacterial expression of the a1/EBP fusion protein.** Phage lysogens were induced in *E. coli* Y1089, and IPTG-induced bacterial lysates were prepared as described by Young and Davis (60) for analysis in gel shift assays. For expression in a plasmid vector, the a1/EBP cDNA was gel purified from the *Not*I-digested Bluescribe plasmid, treated with Klenow polymerase, and ligated into the *Sma*I site of the pGEX 2T vector, so that the cDNA open reading frame was translated as a glutathione-*S*-transferase (GST) fusion protein (55). Fusion protein expression was induced by 1 mM IPTG treatment for 3 h; this procedure was followed by sonication and glutathione-agarose purification of the a1/EBP-GST fusion protein. Protease inhibitors (0.2 ng of leupeptin per ml, 0.2 ng of pepstatin per ml, 1 U of aprotinin HCl per ml, and 1 mM PMSF) were added immediately after sonication. Purified protein was adjusted to 8% glycerol–50 mM NaCl–1 mM EDTA–1 mM DTT, frozen in liquid nitrogen, and stored in aliquots at −70°C.

**Nucleotide sequence accession number.** The sequence data shown in Fig. 7 have been assigned GenBank accession number M95573.

## RESULTS

**Purification of the a1 LTR-binding protein.** The labile a1-binding activity of B cells interacts with regions of the ALV LTR enhancer containing multiple CCAAT/enhancer elements (49, 51). Gel shift analysis of B-cell nuclear proteins with an oligonucleotide probe for the a1 LTR site shows a diffuse ladder of DNA-protein complexes (Fig. 1A). This result could reflect binding to more than one of the overlapping CCAAT/enhancer elements in the a1 oligonucleotide probe, multimerization of complexes, or the binding of several proteins. The a1-binding activity was purified from the S13 bursal lymphoma cell line in order to characterize the protein or proteins involved in labile LTR enhancement. Nuclear extracts from 80 liters of bursal lymphoma cells were initially fractionated by S-300 Sepharose chromatography as described in Materials and Methods. Fractions were assayed for a1-binding activity by gel shift assay with the $^{32}$P-labeled a1 oligonucleotide probe. The a1-binding activity elutes in the included column fractions and is purified two- to threefold by this chromatography, as estimated by comparison of gel shift binding activity per microgram of protein (data not shown).

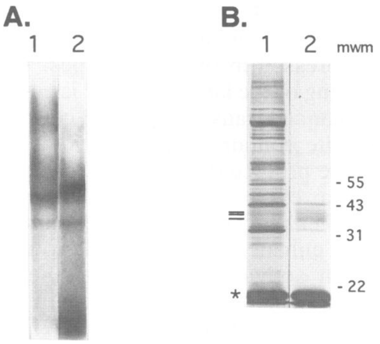

FIG. 1. Analysis of the a1 oligonucleotide affinity-purified a1 LTR-binding protein. (A) Gel shift assay of heat-purified B-cell protein (lane 1) and a1 oligonucleotide affinity-purified protein (lane 2) with the $^{32}$P-labeled a1 oligonucleotide probe. (B) SDS-PAGE and silver staining of heat-purified protein (lane 1) and oligonucleotide-agarose affinity-purified protein (lane 2). Horizontal lines indicate enriched species. The asterisk indicates the carrier lactoglobulin protein. The migration of molecular weight markers (mwm) is indicated in kilodaltons.

The a1-binding activity remains active after heat treatment (49). Therefore, the S-300 fractions were heated at 85°C for 10 min and centrifuged to remove insoluble protein, giving roughly fourfold purification. The heat-purified protein was further enriched by binding to an a1 oligonucleotide-agarose affinity column (24) and elution with 0.6 M KCl. The affinity-purified protein continues to produce a diffuse ladder of LTR-binding activity in gel shift assays with the $^{32}$P-labeled a1 oligonucleotide probe (Fig. 1A). This activity is enriched roughly 4,000-fold relative to the activity of the 0.5 M NaCl nuclear extract.

The composition of the oligonucleotide affinity-purified protein was analyzed by SDS-PAGE and silver staining as described in Materials and Methods. The affinity-purified protein preparation is enriched for three protein species of approximately 35, 40, and 42 kDa relative to the heat-purified protein (Fig. 1B). These species were consistently purified in several independent experiments. Proteins of about 32 and 45 kDa are also observed in the affinity-purified sample, although they are variably present and are not enriched by a1 oligonucleotide-agarose affinity chromatography.

**Characterization of the a1-binding protein.** One or all of the 35- to 42-kDa species purified from bursal lymphoma cells may be specific a1-binding proteins. Proteins were renatured from SDS-polyacrylamide gel slices and tested in gel shift assays to confirm that proteins in this molecular mass range encode a1-binding activity. Heat-purified protein was resolved on a 10% polyacrylamide–SDS gel, and slices of the gel were cut out, eluted, and renatured in buffer containing BSA. Gel shift a1-binding activity is detected in two gel

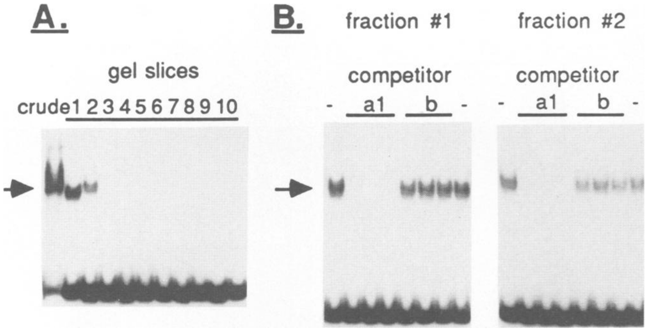

FIG. 2. Identification of a1 LTR-binding proteins after renaturation from SDS-polyacrylamide gel slices. (A) Gel shift analysis of crude heat-purified protein and of protein renatured from slices of an SDS-polyacrylamide gel of heat-purified protein with the $^{32}$P-labeled a1 oligonucleotide probe. The lanes indicate gel slices in the molecular mass range of 20 to 200 kDa. The arrow indicates the DNA-protein complex. (B) Gel shift analysis of protein in gel slice fractions 1 and 2 (molecular mass ranges of 20 to 35 and 35 to 45 kDa, respectively) with the $^{32}$P-labeled a1 oligonucleotide probe. An unlabeled a1 or b oligonucleotide competitor (50-, 100-, or 150-fold molar excess) was added as indicated.

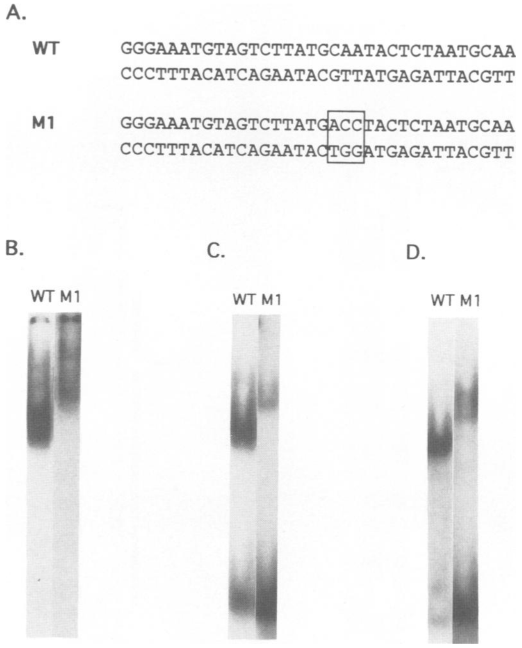

FIG. 3. Binding of purified B-cell protein to LTR CCAAT/enhancer elements. (A) Sequences of the WT and M1 oligonucleotide probes used in gel shift assays. Mutated nucleotides are boxed. (B) Gel shift assay of 0.5 M nuclear extract from bursal lymphoma cells with the $^{32}$P-labeled WT or M1 oligonucleotide probe, as indicated. (C) Gel shift assay of heat-purified protein. (D) Gel shift assay of a1 oligonucleotide affinity-purified protein.

slices which contain 25- to 35-kDa (slice 1) and 35- to 45 kDa (slice 2) proteins (Fig. 2A). The gel shift binding activities from the two slices migrate slightly differently, suggesting that they contain distinct proteins with LTR-binding activity. Both of the binding activities are sequence specific, as judged by gel shift competition with the unlabeled a1 oligonucleotide but not with the unlabeled b oligonucleotide probe (Fig. 2B). The molecular masses of these proteins are within the range of 35 to 42 kDa observed for purified a1 proteins. These data also support the idea that more than one of these species encode a1-binding activity.

**The a1-binding proteins recognize multiple LTR CCAAT/enhancer elements.** The LTR enhancer contains a number of consensus CCAAT/enhancer elements [T(T/G)NNG(C/T)AA(T/G)] recognized by C/EBP (51), and one of these elements is included in the a1 oligonucleotide probe. Three nucleotides in the CCAAT/enhancer element of the wild-type (wt) a1 oligonucleotide probe were mutated in the M1 oligonucleotide probe (Fig. 3A) to determine whether a1-binding proteins recognize this element. The diffuse ladder of crude nuclear extract binding in gel shift assays is altered by this mutation (Fig. 3B). The major rapidly migrating complexes are eliminated, while slowly migrating complexes appear or are accentuated. These binding activities are sequence specific, as judged by gel shift competition assays with homologous or heterologous oligonucleotide probes (data not shown). Heat-purified and oligonucleotide affinity-purified proteins show the same binding pattern (Fig. 3C and D, respectively). These data suggest that the CCAAT/enhancer element is the major recognition sequence for the rapidly migrating a1-binding activity. The M1 gel shift complexes could represent minor or novel binding activities which are visible after removal of the CCAAT/enhancer element-binding activity.

The LTR-binding activity of the oligonucleotide affinity-purified protein was further analyzed by DNase I footprinting with $^{32}$P-labeled ALV LTR sequences (Fig. 4A). A 60-bp region of the LTR enhancer is protected, extending in from the border of the U3 region (−260 bp from the transcription start site; Fig. 4B). Five CCAAT/enhancer elements are contained in this region (Fig. 4C), which could be recognized by the a1-binding proteins. A strong DNase I-hypersensitive site on both strands separates two protected regions (a1 and a2). The a1 site contains the a1 oligonucleotide sequence used for affinity purification of the protein. This footprinting pattern corresponds to that observed for the heat-purified fraction (49), indicating that the same binding activity is maintained through the oligonucleotide affinity purification step. This large protected region could represent multiple binding of one or more proteins.

**Identification of a gene encoding a1 LTR-binding activity.** Further analysis would be facilitated by cloning the gene or genes encoding the a1-binding activity. We used a λgt11 screening technique (54) to identify genes encoding proteins binding to the a1 LTR site. A cDNA library was prepared from S13 bursal lymphoma poly(A)$^+$ RNA and was cloned into the λgt11 vector. Phage expressing *lacZ*-cDNA fusion proteins were screened for binding to $^{32}$P-labeled concatenated a1 oligonucleotide as described in Materials and Methods. One positive clone was identified in a screen of 1.5 × 10$^6$ primary phage, which maintained expression of a1-binding activity through three successive screens (data not shown).

Lysogens of the a1/EBP phage were isolated, and IPTG-induced lysates were prepared and tested for a1 LTR-binding activity by gel shift assays (see Materials and Methods). The lysate contains a single binding activity in gel shift assays with the $^{32}$P-labeled double-stranded a1 oligonucleotide probe, which is specifically competed for by the unlabeled a1 oligonucleotide but not by the b oligonucleotide (Fig. 5A). This a1-binding activity was not observed in lysates from control bacteria (data not shown).

**a1/EBP binds multiple CCAAT/enhancer elements.** The a1 LTR-binding activity of the a1/EBP cDNA clone was further

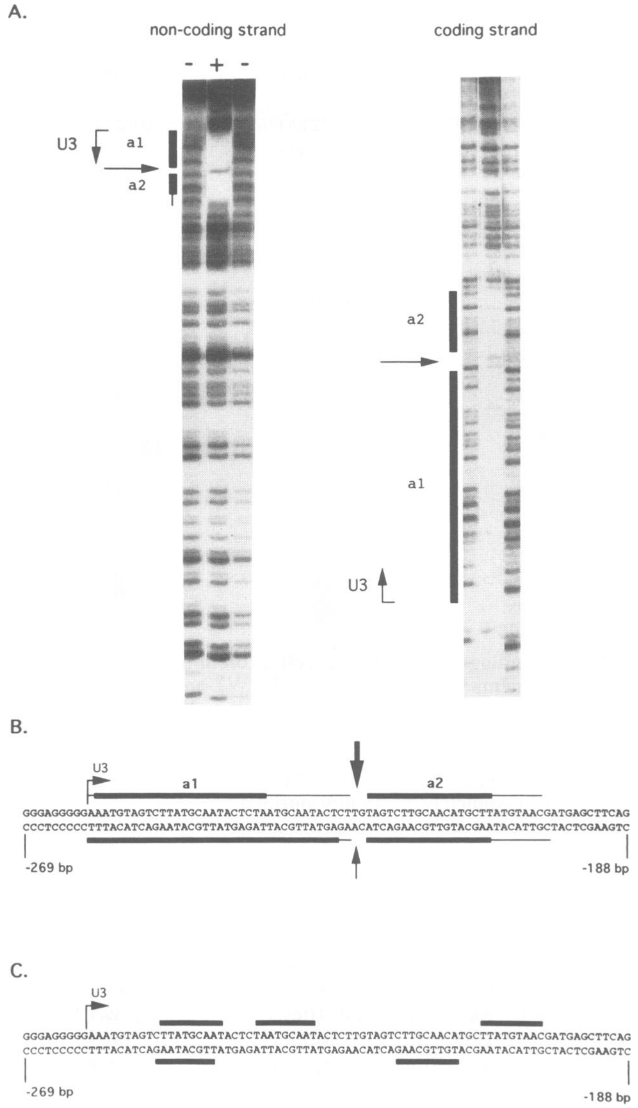

FIG. 4. Binding of purified B-cell protein to two LTR enhancer sites. (A) The LTR probe was $^{32}P$ labeled on the coding or noncoding strand, incubated with (+) or without (−) oligonucleotide affinity-purified a1-binding protein, and digested with DNase I. DNA was purified and resolved on 8 M urea–8% polyacrylamide gels. The bars indicate strongly DNase I-protected sequences, and the lines indicate weakly protected sequences. The 5′ border of the U3 LTR region is indicated. Arrows indicate DNase I-hypersensitive sites. (B) Map of LTR-binding sites of affinity-purified a1-binding protein. The distance from the transcription start site is shown in base pairs. (C) Map of LTR CCAAT/enhancer elements.

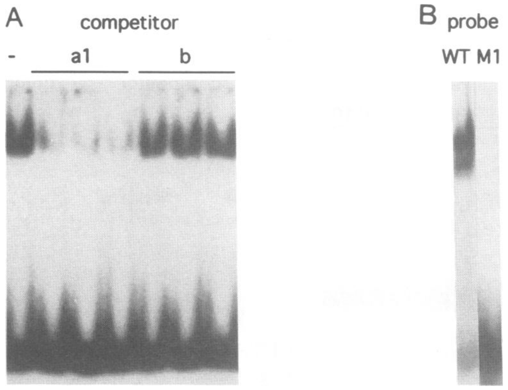

FIG. 5. Binding of a1/EBP to LTR CCAAT/enhancer elements. (A) Lysates of a1/EBPλgt11 lysogens were tested in gel shift assays with the $^{32}P$-labeled a1 oligonucleotide probe. Unlabeled a1 or b competitor (50-, 100-, or 150-fold molar excess) was added as indicated. (B) Purified a1/EBP-GST fusion protein was tested in gel shift assays with the WT or M1 oligonucleotide probe, as indicated.

examined by subcloning the a1/EBP cDNA into the pGEX 2T expression vector, so that the a1/EBP cDNA open reading frame is translated as an a1/EBP-GST fusion protein. Fusion protein expression was induced by IPTG treatment, and the fusion protein was purified from the lysate by glutathione-agarose affinity chromatography (see Materials and Methods). This purified fusion protein also specifically binds to the $^{32}P$-labeled a1 oligonucleotide, as its binding is competed for by the a1 oligonucleotide but not by the b oligonucleotide in gel shift assays (data not shown). We tested the DNA binding specificity of the a1/EBP fusion protein by gel shift assays with the M1 oligonucleotide probe described above (Fig. 3A). Binding of the a1/EBP-GST fusion protein is abolished by mutation of the CCAAT/enhancer element, indicating that this motif is essential for a1/EBP binding (Fig. 5B). The λgt11 a1/EBP–β-galactosidase fusion protein produces a single gel shift complex (Fig. 5A), while the a1/EBP-GST fusion protein produces two complexes (Fig. 5B). The larger β-galactosidase fusion protein may not be able to form multimers, or it may be less susceptible to proteolysis than is the purified a1/EBP-GST fusion protein.

We performed DNase I footprinting experiments with the purified a1/EBP-GST fusion protein to determine whether it binds to more than one of the LTR CCAAT/enhancer elements. The a1/EBP-GST fusion protein protects two regions of the ALV LTR, separated by a strong DNase I-hypersensitive site (Fig. 6). Titration experiments with decreasing amounts of a1/EBP indicate that the a1 and a2 sites are bound with equal affinity (data not shown). Our gel shift data (Fig. 5B) indicate that the a1 CCAAT/enhancer element is required for a1/EBP binding, and it is likely that similar elements are recognized over the a2 site. The protected region is nearly identical to that observed for purified B-cell protein (Fig. 4), supporting the idea that a1/EBP encodes the major a1-binding activity of B cells. This gene could encode any or all of the three purified a1-binding species.

**DNA sequence analysis of a1/EBP cDNA.** The protein-coding potential of the 2.2-kb a1/EBP cDNA was analyzed by primer-directed DNA sequencing (see Materials and Methods). The cDNA contains a 209-amino-acid open reading frame (Fig. 7) which would be translated in the open reading frames expressed in the λgt11 and pGEX 2T expression vectors. The a1/EBP cDNA also contains a long 3′ untranslated region. This cDNA probably does not encode a full-length protein, as the amino terminus does not include an initiator methionine codon. The a1/EBP open reading frame could encode a 26-kDa protein. This appears to be the open reading frame used, as the a1/EBP-GST fusion protein expressed in bacteria is 52 kDa, containing a 26-kDa a1/EBP portion in addition to the 26-kDa GST protein (data not shown).

Computer gene bank library searches indicate that a1/EBP

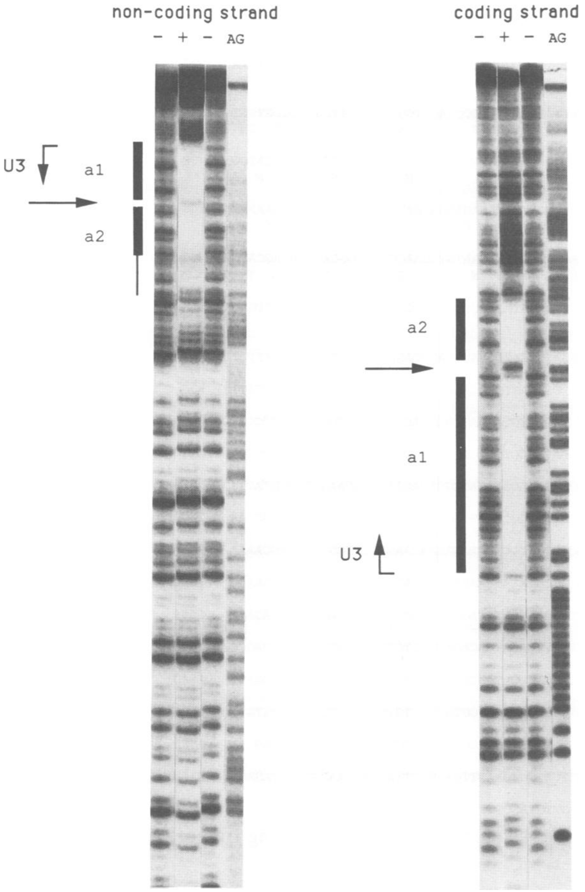

FIG. 6. Binding of a1/EBP to two LTR enhancer sites. $^{32}$P-end-labeled ALV LTR was incubated with (+) or without (−) a1/EBP-GST fusion protein and digested with DNase I. DNA was purified and resolved on 8 M urea–8% polyacrylamide gels along with A+G sequence reactions (AG). The strongly protected sequences are indicated by the heavy bar, and weakly protected regions are indicated by lines. The arrows designate DNase I-hypersensitive sites. The 5′ border of the U3 region is shown.

is encoded by a novel gene, as no closely related sequences are found in the DNA or protein data bases. However, the carboxy terminus of the a1/EBP open reading frame contains amino acid sequence motifs conserved in leucine zipper transcription factors (28). The leucine zipper family is characterized by a carboxy-terminal heptad repeat of leucine residues, which appears to mediate factor dimerization (29). The adjacent basic region is enriched in basic amino acids and is required for DNA binding (29). These sequence motifs were used to align the a1/EBP open reading frame with those of other leucine zipper family members (Fig. 8). The a1/EBP open reading frame is most closely related to that of Ig/EBP (48) and less related to those of C/EBP (28), NF/IL-6 (1), CELF (25), and CRP-1 (58). The putative basic and leucine zipper regions of a1/EBP are nearly identical to those of Ig/EBP, showing 94% amino acid identity (Table 1). The a1/EBP basic region is about 60% identical with that of C/EBP and NF/IL-6, while their leucine zipper regions are different except in the conserved leucines of the four heptad repeats (Fig. 8). Interestingly, all of these proteins bind to CCAAT/enhancer elements, supporting the idea that the conserved basic region specifies DNA binding specificity.

The amino termini of leucine zipper proteins may mediate transcription activation or repression (13, 44). The amino-terminal region of a1/EBP is related to that of Ig/EBP only in the 45 amino acids adjacent to the basic region; it is very different in the amino-terminal 70 amino acids (data not shown), showing overall 39% identity (Table 1). The corresponding amino-terminal regions of C/EBP and NF/Il6 are very different in amino acid composition from a1/EBP. The amino-terminal region of a1/EBP is enriched for basic and acidic residues (Fig. 7), while the corresponding regions of Ig/EBP, C/EBP, and NF/IL-6 are relatively enriched in hydrophobic residues. These data suggest that a1/EBP encodes a novel transcription factor that contains basic and leucine zipper regions closely related to Ig/EBP.

## DISCUSSION

Three a1 LTR-binding proteins of 35 to 42 kDa have been purified from bursal lymphoma cells. Gel slice renaturation experiments confirm that proteins in this molecular mass range encode a1 LTR-binding activity. The close size of these proteins has not allowed us to determine whether one or all of these species encode a1-binding proteins. The a1-binding activity is likely to be associated with more than one of these species, as the gel slice renaturation experiments suggest that at least two different-size proteins bind to a1 LTR sequences in gel shift assays. These multiple species, which retain binding activity, could be due to proteolysis of one a1-binding protein. This possibility is difficult to eliminate (34), even though protease inhibitors were used throughout the purification steps. Alternatively, one a1-binding protein could be modified in a manner that affects its apparent molecular weight, for example by phosphorylation or by processing. Finally, more than one a1-binding protein could be expressed in B cells.

We used a λgt11 cDNA library screening technique to identify a gene encoding an a1-binding protein. This cDNA does not appear to be full length, although it does encode a 209-amino-acid open reading frame, sufficient to encode a 26 kDa protein. Cloning of the full-length a1/EBP cDNA will be required to determine the complete protein-coding potential of this gene and to compare the predicted molecular weight of a1/EBP with those of the purified a1-binding proteins of B cells. We have obtained genomic clones corresponding to the a1 cDNA sequence but thus far have not identified full-length coding sequences. Use of antibodies to a1/EBP will determine whether one or all of the a1-binding proteins purified from B cells are encoded by the a1/EBP gene.

The purified B-cell protein and the a1/EBP fusion protein both bind two LTR enhancer sites in DNase I footprinting experiments and induce a strong DNase I-hypersensitive site between the protected regions. These sites contain several consensus CCAAT/enhancer elements, which appear to be the motifs recognized by these proteins. Similar CCAAT/enhancer elements which are also bound by these proteins are found in the closely related RSV LTR (data not shown). Nuclear extract activities binding these sites in the RSV LTR have been observed in avian fibroblasts (53) and erythroblasts (17), suggesting that a1/EBP or related CCAAT/enhancer element-binding proteins are expressed in many cell types.

The DNase I-hypersensitive site induced by a1/EBP and by B-cell protein binding may correspond to a strong DNase I-hypersensitive site observed in LTR enhancer chromatin from bursal lymphoma cell lines (52). This chromatin hypersensitive site disappears in cells treated with protein synthe-

```
   1  TCCGCTCCCCGCCACAATGTCGGGCAGCGGCGGCGGCAGCAGCGCGGTGAAGAAGAGGAGCCCGGCGGGCTCGGCCTCCTCCATGGCGCAGGAGGAGGAG  100
       S  A  P  R  H  N  V  G  Q  R  R  R  Q  Q  R  G  E  E  E  E  P  G  G  L  G  L  L  H  G  A  G  G  G  E

 101  AAGCAGGCGATGGAGAAGATGCTGGAGGCGGCTGGAGGGGCGCAGGAGAACGGCTGCGCCCGCACGCCTTCTCCCACCGCCGCAAGACATCCCCGCAGAA  200
         A  G  D  G  E  D  A  G  G  G  W  R  G  A  G  E  R  L  R  P  H  A  F  S  H  R  R  K  T  S  P  Q  N

 201  CACCGCTACAGATGCGAACGGAGTAAGCGTGATTCACACCCAGGCGCACAGCAGTGGTTTGCAGCAGGTTCCCCAGCTGGTTCCCGTTAGTCCCGGTGGT  300
        T  A  T  D  A  N  G  V  S  V  I  H  T  Q  A  H  S  S  G  L  Q  Q  V  P  Q  L  V  P  V  S  P  G  G

 301  GGAGGCAAAGCTGTGCCTCCGAGCAAGCAGGGAAAGAAGAATTCCTTTGTGGATAGAAACAGCGATGAGTATCGTCAGCGCAGAGAGCGAAACAACATGG  400
       G  G  K  A  V  P  P  S  K  Q  G  K  K  N  S  F  V  D  R  N  S  D  E  Y  R  Q  R  R  E  R  N  N  M  A

 401  CAGTGAAAAAGAGCCGGTTAAAAAGCAAGCAGAAAGCACAAGACACGCTGCAAAGGGTCACCCAGCTCAAAGAAGAAAATGAACGTTTAGAGGCGAAAAT  500
         V  K  K  S  R  L  K  S  K  Q  K  A  Q  D  T  L  Q  R  V  T  Q  L  K  E  E  N  E  R  L  E  A  K  I

 501  TAAGCTCCTGACCAAGGAGCTGAGCGTACTGAAAGACCTATTCCTTGAGCACGCACACAGTCTCGCAGACAATGTGCAACCTGTTGGCACTGAGAGCACC  600
        K  L  L  T  K  E  L  S  V  L  K  D  L  F  L  E  H  A  H  S  L  A  D  N  V  Q  P  V  G  T  E  S  T

 601  ACAACAAGTGCAGAGAACAGCGGGCAGTAGTCACTGCCACAGCCCGGCGGGATGAACTCTGGAGGTGCAGCCTGTCTGCACCACCCATGCAGGAACTGAG  700
       T  T  S  A  E  N  S  G  Q  *

 701  CAAATGAGGGGTTCTTTTAACCATCGTTGTGTGCGGATCTTTTTAGGTTTAACACTGAAGATTTGATACAATTAAACCAGAAACTGCTTAGGGTATTTCT  800

 801  TTAAAGGCGTTAAATATTTTTCTCCTCCAGAAGATTTGTCTAATAAGTGCAGATCGAAATCCTTAGTCAGCAAGGAGCTTACTATTATGGAAGCAGCATT  900

 901  TCACAGATGTACGCTCACCTTCTTACATTTAGCATAACGGGATAAATTCACCAGGATGCTTCACTGTAGTATCAGAAAGTCATAATTGGGTGAATTCCTT  1000

1001  TAAATACTTTTTAGTGTGTTTAAGCCCTTTCTTTTCTCCATTACATATTACAAACTGCAAAGGCCGTAGAGAGTTTGTTGTCCTGTTTGGTGAACTGCTT  1100

1101  TTCTACCTACAGAGCAATTCCTGCACAGCACCACTTAGGTTTTTCCTATCTCTGGTTGCAGTTAACGAGCAGGGCACAGTAATAGCAAGCTGGGGTACTT  1200

1201  AAAGATGTTTTGCAGAAAACCAGCAGCATTCTGTAGTGATCTTTACAGGCAGTTGGCTTCCACAGGCACGTGGCGGTGTGAAGCACCAAGTATCAGGTAA  1300

1301  AGCAGCAGCAGCAAGGCCTTTTATGCCTGTGTTCATGTTAACCTGGCTTGCACAGTGACCGTGGGGCGGTAGGAGAACAAGATTTTGGTTTCCAGCAAAA  1400

1401  TGTAAAAATGCTACCAACAACCTCTGCTCTGCAAAACTGCATTCCTGCTGGGTGCGGGTATCGGAGAACAAAATACGCAGCTTGGCTGAGCAGCAGAAGG  1500

1501  GGTAGGGCAGCATCTGAACAGCAAGAATGTGTGGATCTCAGCTCACCTTCTTGTTCATTTCCATTCTCACAGCGTCAGATTGCAGCCAGCTGTACAGAAT  1600

1601  TGCACGGTGATTCTAAAACTAACGGCCCTCGTTGAACACCCGAATCAGCAGAATTTCTATATAGCCATCACAGAGTTGTGTGAAGGACGTATCTGCAGGC  1700

1701  TTATTTCCATAGAAGCTTTTCTATAGCAGTTTTTAAAATGCTAGTGATAAGTCTTTGCCATGATCCATAATGCAGTGGTATGTACTATATATTGAGGATT  1800

1801  TTAACAGCAAAAGTAAAGCAGGAACTTTGGCGTACCCTTAATTTATTTTCATTTTAAGTATTTCATTGATGGTATGGTTGTGTTACATTTAACCTGTGTG  1900

1901  TCTGGCTAGTGGATTATTACACTGTCTTAATTTCCCTCCTACACTACCTTTTTCCCGTGTTTTGTAGGATGCTTGTTTGCCTTGTACCTGTACTTAGGAA  2000

2001  AAAAAACCTTGGATATTATCTCTATGAAGATGCTGTTAGAAAATAAAGCCTTTAAAAGTTCTTTCTGAATTTCAATTTTTAATACGAAGGCAACTAGATG  2100

2101  CAGTTTTTTATGGATTGTTTTGGACTATGGTAGTCTTTTTACAAAGATATGTTTTTTTGGTTCTAGCTGC  2170
```

FIG. 7. al/EBP DNA sequence and predicted amino acid sequence of the open reading frame.

sis inhibitors, concomitant with a large decrease in LTR-enhanced c-*myc* transcription (31). This finding supports the hypothesis that binding of al/EBP to the LTR enhancer, as measured by appearance of the DNase I-hypersensitive site, is important for labile LTR transcription enhancement. Interestingly, the al/EBP-binding sites are deleted in endogenous viruses (61), which show low-level LTR-driven transcription and rarely promote lymphomagenesis (6, 46).

DNA sequence analysis of the al/EBP cDNA indicates that it encodes a leucine zipper factor closely related to Ig/EBP, C/EBP, and NF/IL-6 in the conserved putative DNA-binding and leucine zipper dimerization domains. The conservation of the basic-region DNA-binding sequence may reflect the fact that these proteins bind to CCAAT/enhancer elements, including those found in the RSV LTR, which is closely related to the ALV LTR (data not shown) (48, 51). The amino-terminal region of C/EBP encodes transcription-regulatory activities (13, 44). The amino-terminal regions are very different in the various leucine zipper proteins, suggesting that these proteins have different effects on transcription. Interestingly, the amino terminus of al/EBP is enriched in acidic and basic residues, while Ig/EBP and the other leucine zipper proteins are enriched in hydrophobic residues. The carboxy-terminal tails of these proteins

```
                          BASIC REGION

al/EBP    F V D R N S D E Y R Q R R E R N N M A V K K S R L K S K Q K A Q D
              * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Ig/EBP    P M D R N S D E Y R Q R R E R N N M A V K K S R L K S K Q K A Q D
            * *   * *   * * *   * * * * * *   * *   * * *   *   * *
C/EBP     S V D K N S N E Y R V R R E R N N I A V R K S R D K A K Q R N V E
            * *     * * * *     * * * * * *   * *   * * *   *   *
NF/IL6    T V D K H S D E Y K I R R E R N N I A V R K S R D K A K M R N L E

                          LEUCINE ZIPPER

                        L             L             L             L
al/EBP    T L Q R V T Q L K E E N E R L E A K I K L L T K E L S V L K D L F L E H A H S L A D N V Q P V
          * * * * *   * * * * * * * * * * * * * * * * *   * * * * * * * * * * * * * * * * * * * * * *
Ig/EBP    T L Q R V N Q L K E E N E R L E A K I K L L T N E L S V L K D L F L E H A H S L A D N V Q P I
          *   *   *     *       *   * *             *     * *     *       *           *
C/EBP     T Q Q K V L E L T S D N D R L R K R V E Q L S R E L D T L R G I F R Q L P E S S L V K A M G N
          *       *     *     * * * * *     *       *     * * *   *     * *             *
NF/IL6    T Q H K V L ¯ L T A E N E R L Q K K V E Q L S R E L S T L R N L F K Q L P E P L L A S S G H C
```

FIG. 8. Comparison of the al/EBP protein sequence with sequences of other leucine zipper proteins. The putative basic and leucine zipper regions of al/EBP were aligned with those of Ig/EBP, C/EBP, and NF/IL-6 (1, 28, 48). Conserved leucine residues are highlighted. Asterisks indicate regions of amino acid identity with al/EBP.

TABLE 1. Percent amino acid identity of al/EBP with regions of other leucine zipper proteins[a]

| Protein | % Amino acid identity | | |
|---|---|---|---|
| | Amino terminus (114) | Basic region (33) | Leucine zipper (47) |
| Ig/EBP[b] | 39 | 94 | 94 |
| C/EBP[c] | 6 | 64 | 28 |
| NF/IL-6[d] | 11 | 58 | 36 |

[a] Alignment of putative basic and leucine zipper regions was based on comparison with C/EBP. The number of amino acids compared is given in parentheses.

[b] Data from Roman et al. (48).

[c] Data from Landshulz et al. (28).

[d] Data from Akira et al. (1).

are also very different, as the 15-amino-acid tail of al/EBP is only slightly related to the 10-amino-acid tail of Ig/EBP. The al/EBP gene is also different from the Ig/EBP gene in that the al/EBP protein appears to be 35 to 42 kDa, while Ig/EBP is 45 kDa (48). Ig/EBP forms heterodimers with C/EBP in vitro, suggesting that combinations of leucine zipper proteins may modulate transcription (48). Further experiments will determine whether an Ig/EBP homolog is expressed in avian B cells and whether it can dimerize with al/EBP.

We have previously found that the labile a1, a3, and/or b* proteins appear to be essential for LTR-enhanced c-*myc* hyperexpression, and these labile proteins may play an important role in the susceptibility of pre-B cells to ALV lymphomagenesis. We do not yet know whether the protein encoded by the al/EBP gene is labile. However, use of the al/EBP DNA clones will allow us to generate antibodies to al/EBP to determine whether it encodes the labile binding activity of B cells. Computer analysis of the al/EBP open reading frame indicates several motifs that could regulate labile expression. The amino-terminal region contains a PEST consensus sequence, thought to target rapid protein turnover (47). The al/EBP protein also contains a number of consensus creatine kinase II, cyclic AMP-dependent kinase, and protein kinase C motifs (26). Labile phosphorylation of al/EBP could affect its transcription-regulatory activity, as phosphorylation of a number of transcription factors has been shown to affect their DNA-binding activities (4, 32). Further experiments will reveal whether al/EBP is labile, how this lability is regulated, and the role of this lability in ALV tumor susceptibility of immature bursal cells.

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